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Laser Nephelometric Determination of Glycosaminoglycans – Method and Application

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Summary: Light scattering due to the formation of insoluble complexes between the long-chain quaternary ammonium salt N-cetylpyridinium chloride and glycosaminoglycans was utilized for a relative simple, sensitive and precise determination of total and specific types of glycosaminoglycans by laser nephelometry. The addition of the ammonium salt to solutions of various glycosaminoglycans in 0.03 mol/l NaCl produces a time-dependent increase in light scattering, which reaches a maximum between 14 and 18 h of complex formation, irrespective of the type of glycosaminoglycan studied. Only keratan sulphate does not generate light scattering, and is therefore not detectable by the procedure. The scattering of laser light by certain types of sulphated glycosaminoglycans (e.g. heparan sulphate, heparin) depends more on the degree of sulphation (charge density) than on chain length within a certain range. Optimum light scattering was found at 28 mmol/l N-cetylpyridinium chloride and at a ionic strength around 0.03 mol/l NaCl.

The detection limits and linear ranges of the individual glycosaminoglycans were evaluated. For the determination of chondroitin sulphate, laser nephelometry is at least 8 times more sensitive and much more simple than the modified carbazole method (glucuronic acid). The intra-assay and inter-assay coefficients of variation are about 4% and 7%, respectively. Laser nephelometry is much more sensitive than turbidimetry. Complex synthetic mixtures of glycosaminoglycans and biological fluids were accurately differentiated by successive chemical and enzymatic degradation of the respective glycosaminoglycans followed by the measurement of the resulting reduction of laser light scattering. In synovial fluids from non-inflammatory joint diseases, light scattering (units/ml) was about 4.5 times higher than in synovial fluids from inflammatory articular lesions. In both pathologic conditions nearly all of the light scattering can be attributed to hyaluronic acid.

Lasernephelometrische Bestimmung der Glykosaminoglykane – Methode und klinische Anwendung

Zusammenfassung: Lichtstreuung durch Bildung unlöslicher Komplexe zwischen dem langkettigen quaternären Ammoniumsalz N-Cetylpyridiniumchlorid und Glykosaminoglykanen wurde für eine relativ einfache, empfindliche und präzise Bestimmung der gesamten und speziellen Glykosaminoglykane mittels Lasernephelometrie nutzbar gemacht. Die Zugabe des Ammoniumsalzes zu Lösungen verschiedener Glykosaminoglykane in 0,03 mol/l NaCl erzeugt einen zeitabhängigen Anstieg des Streulichtes, welches ein Maximum 14 bis 18 Stunden nach Komplexbildung erreicht, unabhängig von dem jeweiligen Typ der Glykosaminoglykane. Nur Keratansulfat erzeugt keine Streulichtzunahme und ist somit nicht nachweisbar. Für die Eigenschaft der Laserlichtstreuung bestimmter sulfatierter Glykosaminoglykantypen (z. B. Heparansulfat, Heparin) ist der Grad der Sulfatierung (Ladungsdichte) kritischer als Variationen ihrer Kettenlänge innerhalb eines gewissen Bereiches.

Optimale Lichtstreuung wurde gefunden bei 28 mmol/l N-Cetylpyridiniumchlorid und bei einer Ionenstärke um 0,03 mol/l NaCl. Die Nachweisgrenzen und linearen Bereiche der individuellen Glykosaminoglykane wurden bestimmt. Im Vergleich zur Bestimmung des Chondroitinsulfates durch die modifizierte Carbazol-

reaktion (Glucuronsäure) erweist sich die Lasernephelometrie als mindestens 8mal empfindlicher und wesentlich einfacher. Die Variationskoeffizienten der Bestimmungen in der Serie und von Tag zu Tag betragen jeweils 4% und 7%. Im Vergleich zur Turbidimetrie ist die Lasernephelometrie wesentlich empfindlicher. Komplizierte synthetische Gemische von Glykosaminoglykanen und biologische Flüssigkeiten können zuverlässig durch aufeinanderfolgende chemische und enzymatische Degradation der Glykosaminoglykane, gefolgt von der Messung der resultierenden Abnahme der Laserlichtstreuung, differenziert werden. Durch Anwendung dieses analytischen Prinzips wurden in Synovialflüssigkeiten nicht-entzündlicher Gelenkergüsse etwa 4,5 mal soviel Streulichteinheiten wie in jenen entzündlicher Gelenkerkrankungen festgestellt. Unter beiden pathologischen Bedingungen ist nahezu das gesamte Streulicht auf Hyaluronsäure zurückzuführen.

Introduction

Glycosaminoglycans are complex linear (unbranched) heteropolysaccharides which are covalently linked to a core protein forming a high molecular weight proteoglycan (1—4). With the exception of hyaluronic acid, which is not covalently bound to a protein moiety, the components of the serially repeating disaccharide unit are substituted with sulphate residues, thus giving the total molecule a highly anionic charge. Due to variations in the composition of the disaccharide unit (usually N-acetylglucosamine, N-acetylgalactosamine and glucuronic acid, iduronic acid), in the charge density, in the distribution of sulphate residues between glucuronic acid and hexosamine, and in the chain length (molecular weight), glycosaminoglycans comprise a greatly heterogeneous class of carbohydrates with a high degree of molecular polydispersity (5).

Glycosaminoglycans are main constituents of the intercellular matrix in tissues such as cartilage, skin and cornea (4) but are also present in parenchymatous organs like liver (6), brain (7, 8), and kidney (9, 10). In the latter type of tissues glycosaminoglycans are also distributed intracellularly (10, 12) and pericellularly (9, 13, 14). This subcellular localization suggests that glycosaminoglycans may have some as yet unknown regulatory role, in addition to their mechanical functions in the intercellular compartment. The presence of glycosaminoglycans not only in tissues but also in several body fluids, e.g. serum (15—17), urine (18), amniotic fluid (19), and synovial fluid (20) has been well documented. Although characteristic changes of the total amount and, more seldom, of the pattern of glycosaminoglycans in serum and urine have been described in acquired (21—25) and hereditary (26—29) diseases, our present knowledge of the possible pathobiochemical or diagnostic significance of these alterations is limited. This failure is partly due to the difficulties inherent in the methods of determination.

The structural diversity of glycosaminoglycans and some of their structural similarities to glycoproteins have seriously complicated the development of analytic procedures with both sensitivity and specificity for the individual types of glycosaminoglycans. Whereas ion-exchange chromatographic separation and differential extraction or fractionation of glycosaminoglycans are valuable research tools (30) these methods are too laborious for application in the routine laboratory. Electrophoresis of glycosaminoglycans, using a one-dimensional (31—33), or two-dimensional system (34, 35) has been suggested as a method suitable for routine analysis, but incomplete separation of the bands partly due to the heterogeneity of individual glycosaminoglycans and species variation between tissue or fluid and reference glycosaminoglycans are frequent limiting factors in the identification of glycosaminoglycans. For routine purposes the formation of insoluble complexes between anionic polysaccharides and a variety of cationic substances has been utilized in a great number of modifications (30, 36). The glycosaminoglycan-precipitating ability of the cationic dye Alcian Blue 8GX (37) has been exploited for quantitative measurement of glycosaminoglycans in urine (38, 39) and serum (40), respectively, but the specificity of the method in complex biological fluids is limited (41).

The introduction of long-chain quaternary ammonium salts such as N-cetylpyridinium chloride and cetyltrimethylammonium bromide (42, 43) as precipitating agents of glycosaminoglycans has greatly facilitated the development of relatively simple and reliable procedures for their quantitation and isolation. Both quaternary ammonium salts have been applied for the measurement of glycosaminoglycans in serum (45, 46) and urine (22, 23, 24, 44), respectively. A further improvement of practicability was achieved by utilizing the formation of relatively insoluble complexes between cetyltrimethylammonium bromide and N-cetylpyridinium chloride, respectively, and glycosaminoglycans in a turbidimetric assay of the latter in body fluids (47—49). Although this analytic principle offers a convenient method for the determination of glycosaminoglycans, the relatively

low sensitivity proves to be disadvantageous. In the present study, we have used this method of complex formation, and we have combined the advantage of a homogeneous assay system with the sensitivity provided by laser nephelometry for the measurement of glycosaminoglycans in fluids.

Individual glycosaminoglycans were identified by their selective chemical and enzymatic destruction. A preliminary description of the method has been given elsewhere (50). After completion of the present studies, *Warren* and *Manley* (51) reported on a laser nephelometric measurement of glycosaminoglycans in human serum.

Materials and Methods

Chondroitin AC lyase (EC 4.2.2.5, lot E 0602) and chondroitin ABC lyase (EC 4.2.2.4, lot E 8804) were purchased from Seikagaku Kogyo Comp., Tokyo, Japan; a crude fraction of *Streptomyces* hyaluronate lyase (EC 4.2.2.1, Op 166 I/II, 500 U/mg protein) was kindly provided from Behring Werke AG, Marburg, FRG; N-cetylpyridinium chloride (monohydrate) was from E. Merck AG, Darmstadt, FRG; Arteparon (glycosaminoglycan polysulphate, a chemically oversulphated chondroitin sulphate, lot. 79204 79) was obtained from Luitpold Werke, Munich, FRG. High molecular weight calf thymus DNA (lot. 737 5409) and RNA (a mixture of 16S and 23S ribosomal RNA from *E. coli* MRE 600, lot. 135 7403) were purchased from Boehringer GmbH, Mannheim, FRG. The natural glycosaminoglycans were purified from the following sources:
chondroitin sulphate from bovine tracheal cartilage (52),
dermatan sulphate from pig skin (53),
keratan sulphate from pig cornea (54),
heparan suphate (Upjohn Comp. Kalamazoo, USA) from pig mucosa was repurified and separated by Dowex chromatography into low and highly sulphated subfractions with sulfate/glucosamine molar ratios of 0.31 and 2.40, respectively;
heparin (Upjohn Comp.) from pig mucosa, with a sulfate/glucosamine molar ratio of 1.87, was separated into a low (<4000) and a high molecular weight fraction; hyaluronic acid was isolated from human synovial fluid.

Preparation of samples

Synovial fluids were obtained by puncture from diseased joints. The condition of the diseased joints was classified as inflammatory or non-inflammatory according to cytological, clinical-chemical and physico-chemical criteria (55, 56). To remove the cells the synovial fluids were centrifuged for 15 min, 1500 *g*, 25 °C and the cell-free supernatant kept at −20 °C until analysis was performed. The material was diluted 1 : 40 with 0.03 mol/l NaCl and filtered through Millex filters before N-cetylpyridinium chloride was added as described below.

Determination of purified glycosaminoglycans by laser light scattering

Unless otherwise stated the principle procedure for the measurement of glycosaminoglycans was as follows. Highly concentrated stock solutions (1 g/l) of the various glycosaminoglycans in 0.03 mol/l NaCl were prepared and diluted geometrically with 0.03 mol/l NaCl to obtain the final concentrations. The various dilutions were filtered through Millex disposable filters (Millipore GmbH, Neu-Isenburg, FRG) and the light scattering, normally between 0.06 and 0.08 V, was measured. N-cetylpyridinium chloride was used as a 0.28 mol/l solution in 0.03 mol/l NaCl and filtered as above. Light scattering of this solution was lower than 0.03 V.

200 µl of the solutions of glycosaminoglycans were mixed in LN-cuvettes (Behring Werke AG, Marburg, FRG) with 0.1 volume (20 µl) of the N-cetylpyridinium chloride solution and incubated for various times, routinely 24 h, at room temperature. Before measurement the mixture was thoroughly whirled. The intensity of light scattering (voltage, V) was determined in a helium-neon laser nephelometer (wave length 632.8 nm, Behring Werke AG, Marburg) equipped with an automatic cuvette transport system and a Hewlett-Packard 9815 A microcomputer. For the final calculation light scattering of blank incubations was subtracted from those of the complete N-cetylpyridinium chloride-glycosaminoglycan mixture.

Determination of specific glycosaminoglycans in synthetic mixtures of purified glycosaminoglycans and in body fluids by laser light scattering

Mixtures of different glycosaminoglycans, of which the concentration and the laser light scattering of the individual compounds were known, were prepared in 0.03 mol/l NaCl and subjected to chemical and enzymatic treatment as described previously (57).

For estimation of N-sulphated glycosaminoglycans (heparan sulphate, heparin) the assay, total volume 1.20 ml, contained 1.0 ml glycosaminoglycan solution, 0.1 ml of 1.5 mol/l $NaNO_2$ and 0.1 ml 1 : 5 diluted glacial acetic acid. The control incubation had a similar composition but $NaNO_2$ was omitted. Light scattering by the blank incubations (without added cetylpyridinium chloride) of both assay and control was similar and lower than 0.1 V; it was therefore neglected. The mixtures were incubated for 80 min at 37 °C, heated (100 °C, 1 min), cooled and centrifuged (4 min, 2000 *g*). 0.12 ml N-cetylpyridinium chloride solution was added to the filtered supernatant, mixed and used for light scattering measurement as described above.

The fraction of heparan sulphate and heparin in the mixture was calculated from the difference in the light scattering intensity between assay and control incubation.

The proportion of chondroitin sulphate in the mixtures was determined by degradation with chondroitin AC lyase (EC 4.2.2.5) (58). The assay, total volume 0.60 ml, contained 0.3 ml glycosaminoglycan solution, 0.1 ml "enriched" Tris-HCl buffer, pH 8.0 and 0.2 ml (1 unit) chondroitin AC lyase. The proportion of chondroitin sulphate + dermatan sulphate was determined similarly, but the assay contained 0.2 ml (1 unit) of chondroitin ABC lyase (EC 4.2.2.4) instead of AC lyase. The control incubations were composed similarly but enzymes were omitted. Blank incubations (without N-cetylpyridinium chloride) of both assay and control gave similar, low light scattering (<0.1 V), and were therefore neglected. After incubation for 3 h at 37 °C the mixtures were heated at 100 °C for 1 min, cooled, centrifuged for 4 min at 2000 *g* and the supernatant filtered through Millex filters. After addition of 0.06 ml N-cetylpyridinium chloride solution light scattering was measured as above.

The fraction of chondroitin sulphate + dermatan sulphate was calculated from the difference in the light scattering between the ABC lyase assay and the control incubation. The fraction of chondroitin sulphate was calculated from the respective difference between the AC lyase assay and control incubation, and the fraction of dermatan sulphate was calculated from the difference in the light scattering between the AC-lyase and ABC-lyase assay of which the control incubations are identical. It should be noted that due to the partial degradation of hyaluronate by chondroitin lyases the procedure is only valid if hyaluronate is either absent from the mixture or has been removed by digestion with hyaluronate lyase.

The portion of hyaluronic acid in the mixture of glycosaminoglycans was measured by selective destruction of the glycosaminogly-

can with hyaluronate lyase (EC 4.2.2.1) (57, 59). The assay, total volume 1.5 ml, contained 0.5 ml glycosaminoglycan solution, 0.5 ml 0.1 mol/l sodium acetate, pH 5.0 and 0.5 ml hyaluronate lyase (250 units).

The control incubation did not contain hyaluronate lyase but otherwise had a similar composition. The respective blank incubation was omitted. The mixtures were incubated for 20 h at 37 °C and processed as described for chondroitin sulphate. After addition of 0.15 ml N-cetylpyridinium chloride solution the intensity of scattered light was recorded as mentioned above. The fraction of hyaluronic acid was calculated from the difference in the light scattering between assay and control incubation.

If the concentrations (mg/l) rather than the relative fractions of specific glycosaminoglycans are required, the intensity of light scattering of the individual glycosaminoglycans determined as above is refered to a calibration curve of known concentrations of the respective, highly purified type of glycosaminoglycan. The specific types used as references are described in the legends to the tables and figures. Special care was taken to ensure that light scattering was in the linear range of glycosaminoglycan concentration by measuring various dilutions in 0.03 mol/l NaCl of the glycosaminoglycan-containing samples, e.g. urine, synovial fluid and arteficial mixtures.

Determination of glycosaminoglycans by turbidimetry

The solution of glycosaminoglycans and the incubation with N-cetylpyridinium chloride were exactly as described for nephelometry. The turbidity of the glycosaminoglycan-N-cetylpyridinium chloride mixture was read at 340 nm using a centrifugal fast analyzer (GEMSAEC, Electro Nucleonics, USA). Only the absorbance of the first reading was recorded because subsequent readings (reading interval 60 s) produced false low absorbance values due to a sedimentation of the glycosaminoglycan-N-cetylpyridinium chloride complex. All measurements were performed at least in triplicate.

Other analytical procedures

Uronic acid was determined according to the *Bitter & Muir* modification (60) of the carbazole reaction introduced by *Dische* (61). The analysis was mechanized by adaptation to a Technicon Autoanalyzer II system.

Results

Characterization of the glycosaminoglycan-N-cetylpyridinium chloride complex formation by laser light scattering measurements

The addition of N-cetylpyridinium chloride to various solutions of specific glycosaminoglycans in 0.03 mol/l NaCl produces a time dependent increase in laser light scattering (fig. 1). The rate of increase is influenced by the concentration of glycosaminoglycan because being higher for more concentrated solutions (fig. 1b, c). Although the time-course varies slightly between the various types of glycosaminoglycans the maximum intensity of scattered light was recorded between 14 and 18 h of complex formation, independent of the type and the concentration of the glycosaminoglycan studied (fig. 1).

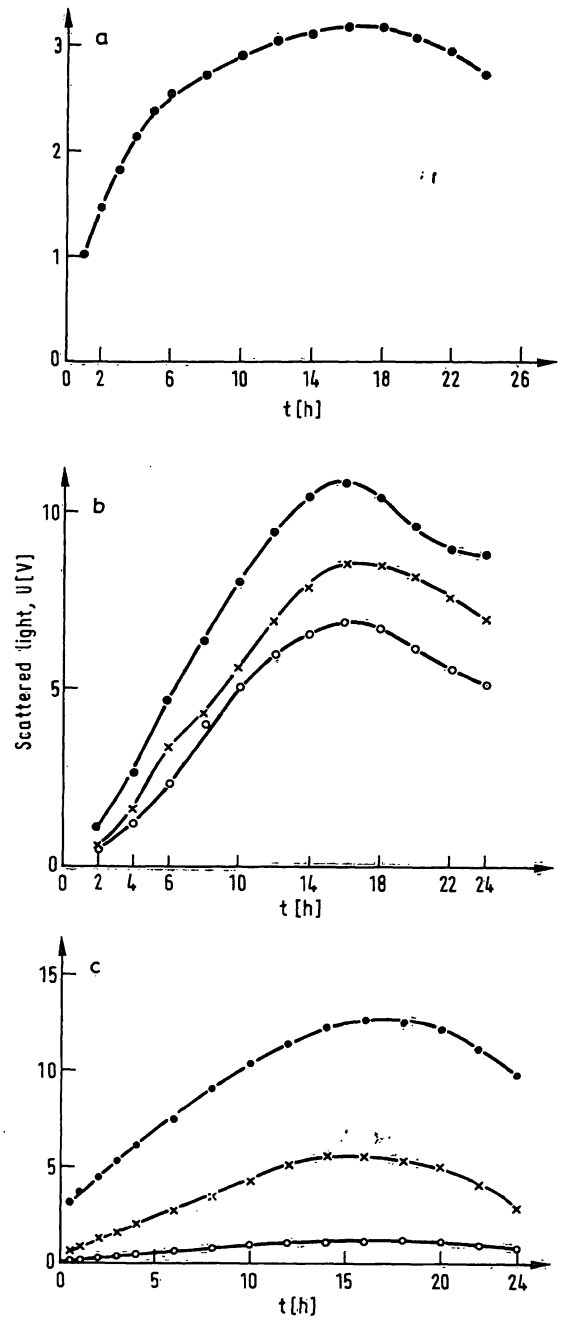

Fig. 1. Time course of the complex formation between various glycosaminoglycans and N-cetylpyridinium chloride monitored by laser light scattering measurements. The conditions were as described in fig. 2
a) highly sulphated heparan sulphate (125 mg/l),
b) glycosaminoglycan polysulphate (Arteparon) in concentrations (mg/l) of 125 (●——●), 62.5 (×——×), 31,25 (O——O),
c) chondroitin sulphate in concentrations (mg/l) of 125 (●——●) 31.3 (×——×) and 7.8 (O——O).

Significant differences in the concentration dependency of laser light scattering were found between the specific types of glycosaminoglycans (figs. 2, 3, 4). Chondroitin sulphate develops a more intense light scattering than dermatan sulphate whereas keratan sulphate is unable to produce light scattering in the presence of N-cetylpyridinium chloride (fig. 2).

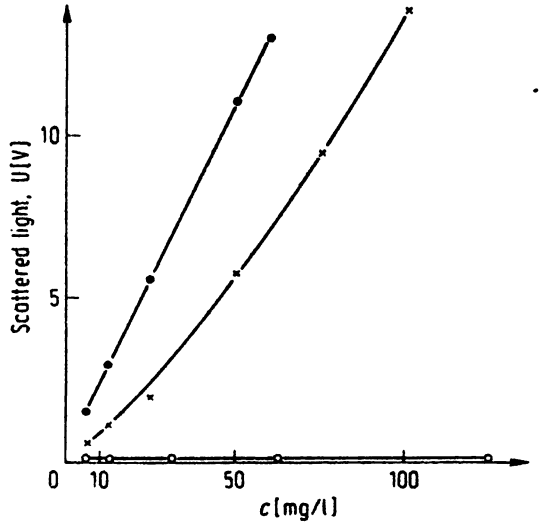

Fig. 2. Laser light scattering of the N-cetylpyridinium chloride-glycosaminoglycan complex as function of variable concentrations of chondroitin sulphate (●——●), dermatan sulphate (×——×) and keratan sulphate (○——○). Increasing concentrations of glycosaminoglycans in 0.03 mol/l NaCl were incubated for 24 h with N-cetylpyridinium chloride. Complex formation was recorded by measuring the intensity of scattered laser light.

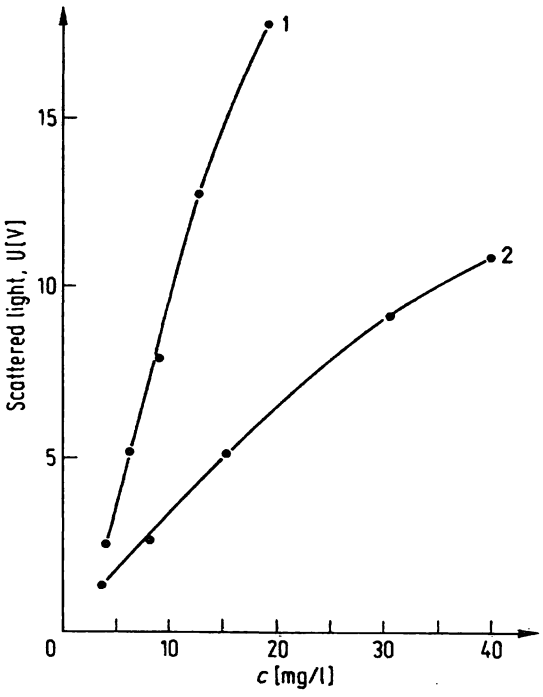

Fig. 3. Laser light scattering of the N-cetylpyridinium chloride-glycosaminoglycan complex as function of variable concentrations of hyaluronic acid (1) and glycosaminoglycan polysulphate (2, Arteparon).
The conditions were as described in figure 2.

Thus, the latter type of glycosaminoglycan cannot be measured by the laser nephelometric method. The intensity of light scattering is independent of the presence or absence of sulphate groups in the carbohydrate chain. This is documented in figure 3 which shows for hyaluronic acid a much stronger dose-dependent increase of light scattering (1 in

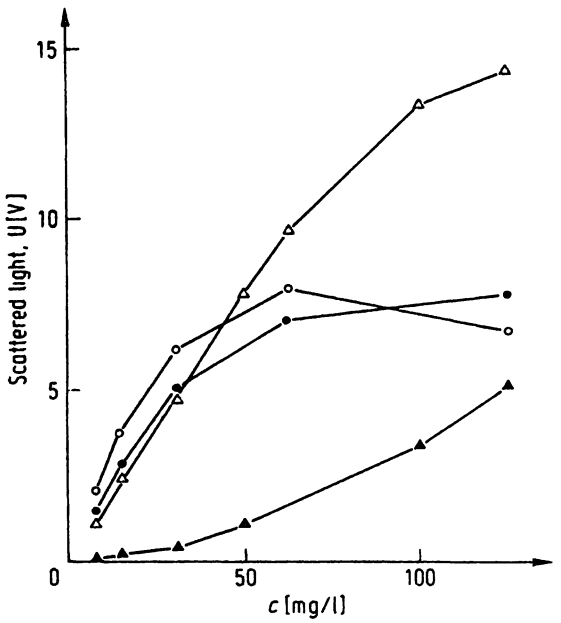

Fig. 4. Laser light scattering of the N-cetylpyridinium chloride-glycosaminoglycan complex as function of variable concentrations of high-sulphated heparan sulphate (△——△, sulphate/hexosamine ratio 2.40), low-sulphated heparan sulphate (▲——▲, sulphate/hexosamine ratio 0.31), high molecular weight heparin (○——○, sulphate/hexosamine ratio 1.87), and low molecular weight heparin (●——●, sulphate/hexosamine ratio 1.86).
The conditions of the assay were as described in figure 2.

fig. 3) than for glycosaminoglycan polysulphate (2 in fig. 3), a chemically over-sulphated chondroitin sulphate with a sulphate/hexosamine molar ratio of 4.0. However, different degrees of sulphation of heparan sulphate influence significantly the development of laser light scattering in the presence of N-cetylpyridinium chloride (fig. 4). Similar concentrations of a higher sulphated subfraction (sulphate/hexosamine molar ratio 2.4) produces much more light scattering than the lower sulphated fraction (sulphate/hexosamine molar ratio 0.31) (fig. 4). Two types of heparin, differing in the chain length but having identical sulphate/hexosamine molar ratios of 1.87, generate similar light scattering curves (fig. 4). Thus, for the property of scattering laser light the degree of sulphation of a certain type of glycosaminoglycan seems to be more important than certain variations of the lengths of the carbohydrate chain.

Although the final concentration of N-cetylpyridinium chloride in the assay, routinely 28 mmol/l, can be varied over a wide range without affecting the intensity of scattered light (tab. 1), care has to be taken that the concentration of the ammonium salt is in excess of that of the glycosaminoglycans. Otherwise falsely low concentrations of glycosaminoglycans may be recorded, due to reduced light scattering.

Tab. 1. Effect of variable concentrations of N-cetylpyridinium chloride on the intensity of laser light scattering produced in the presence of 22.5 mg/l glycosaminoglycan polysulphate (Arteparon).
The reaction was performed in 0.03 mol/l NaCl, the incubation period was 24 h.

Concentration of N-cetylpyridinium chloride (mmol/l)	Intensity of scattered light (V)
280	7.0
140	7.0
28	7.0
14	7.0
2.8	6.5
1.4	5.9

With respect to the ionic conditions, optimal light scattering was produced in approximately 0.03 mol/l NaCl. In 0.3 mol/l NaCl the light scattering was not reproducible due to a non-homogeneous suspension of the insoluble glycosaminoglycan-N-cetylpyridinium chloride complexes (tab. 2).

Tab. 2. Effect of variable concentrations of NaCl on laser light scattering in the presence of 22.5 mg/l glycosaminoglycan polysulphate and 0.028 mol/l N-cetylpyridinium chloride. The mixture was incubated for 24 h.

Concentration of NaCl (mol/l)	Intensity of scattered light (V)
0.30	turbidity inhomogeneous
0.10	6.2
0.03	7.0
0.01	6.9

In table 3 the linear ranges and detection limits of the laser nephelometric determinations of various, purified glycosaminoglycans are listed. The detection limit is defined as the result which is clearly different from the matrix (a solution of 28 mmol/l N-cetylpyridinium chloride in 0.03 mol/l NaCl), defined as 3 standard deviations of the appropriate blank value (62). If based on a detection limit of the carbazole reaction (60) of about 10 µmol/l uronic acid and an uronic acid content of chondroitin sulphate of 1.3 µmol/mg, the laser nephelometric detection limit of 1 mg/l chondroitin sulphate (tab. 3) corresponds to an uronic acid concentration of 1.3 µmol/l. Thus, laser nephelometry proves to be about 8 times more sensitive than quantitation of chondroitin sulphate by the carbazole method. Taking into account a minimum filling volume of the laser nephelometry cuvette of 0.2 ml, laser nephelometry enables the precise determination of an amount of glycosaminoglycan equivalent to 0.25 nmol uronic acid. As already mentioned keratan sulphate is not measurable by to procedure.

Tab. 3. Detection limits and linear ranges of the laser nephelometric determination of various glycosaminoglycans.
The glycosaminoglycans described in "Materials" were dissolved in 0.03 mol/l NaCl. The detection limit, defined as the analytical result which is clearly detectable and different from the blank value (3 standard deviations of the blank value) was assessed by successive dilutions of a stock solution. Addition of N-cetylpyridinium chloride and incubation were as described in "Methods".

Type of glycosaminoglycan	Detection limit (mg/l)	(scattered light, V)	Linear range (mg/l)
Chondroitin sulphate	1	0.7	1–100
Dermatan sulphate	5	0.4	5–125
Heparin (low molecular weight)	8	0.7	8– 30
Heparin (high molecular weight)	5	0.6	5– 30
Heparan sulphate (highly sulfated)	5	0.3	5– 90
Hyaluronic acid	3.5	0.5	3.5– 20
Keratan sulphate	–	–	–

Results of precision control analyses of the determination of various concentrations of chondroitin sulphate by the laser nephelometric method are summarized in table 4.

Tab. 4. Intra-assay and inter-assay precision of the laser nephelometric measurement of chondroitin sulphate from bovine tracheal cartilage. The glycosaminoglycan was used in 3 different concentrations (62.5 mg/l, 45.0 mg/l, 25.0 mg/l), the determination was performed as described in "Methods".

	Intra-assay precision			Inter-assay precision		
Number of determinations	20	20	20	10	10	10
Light scattering mean (V)	14.73	9.90	5.47	14.38	10.06	4.93
S.D. (V)	0.66	0.34	0.25	1.03	0.55	0.48
Coefficient of variation (%)	4.4	3.4	4.5	7.1	5.4	9.7

Laser nephelometrically recorded differentiation of arteficial mixtures of glycosaminoglycans by successive degradation with nitrous acid, hyaluronate lyase, and chondroitin lyase

An application of the laser nephelometric principle to the determination of the composition of glycosaminoglycans in a variety of biological fluids necessitates the differentiation between the various types of glycosaminoglycans. Therefore, a discriminating method was developed which is based on the relative

Tab. 5. Recovery of individual glycosaminoglycans in a synthetic mixture of 4 specific, highly purified glycosaminoglycans by chemical and enzymatic treatment followed by laser light scattering measurements.
A solution containg dermatan sulphate (40 mg/l), chondroitining sulphate (10 mg/l), heparan sulphate (10 mg/l), and hyaluronic acid (5 mg/l) in 0.03 mmol/l NaCl was prepared. The light scattering of each type of glycosaminoglycan at the respective concentration was determined separately to obtain the fraction of total light scattering due to a specific type. Then, the mixture was sequentially degraded with nitrous acid, hyaluronate lyase and chondroitin AC/ABC lyases to measure the scattered light due to heparan sulphate, hyaluronic acid, chondroitin sulphate, and dermatan sulphate, respectively, as described in "Methods". The type-specific scattered light measured is expressed as fraction (recovery) of that determined for the respective glycosaminoglycan separately. The mean values ± S.D. of 5 determinations are listed.

Type of glycosaminoglycans	Concentrations in the mixture (mg/l)	Scattered light expected (V)	Scattered light measured (V)	Mean recovery (fraction of expected scattered light)
Dermatan sulphate	40.0	4.5	4.28 ± 0.20	0.95
Chondroitin sulphate	10.0	6.5	6.43 ± 0.03	0.99
Heparan sulphate	10.0	1.5	1.48 ± 0.05	0.99
Hyaluronic acid	5.0	7.0	7.06 ± 0.06	1.01
Total	65.0	19.5	19.25 ± 0.09	0.99

reduction of light scattering obtained by degradation of N-sulphated glycosaminoglycans, i.e. heparin and heparan sulphate, with nitrous acid, by degradation of hyaluronic acid with hyaluronate lyase, and by degradation of chondroitin sulphate and dermatan sulphate with chondroitin AC and ABC lyases. Due to the limited specificity of chondroitin lyases, which also degrade to a certain extent hyaluronate, the digestion of the latter type of glycosaminoglycan with hyaluronate lyase should be performed before the chondroitin lyases are used. Hyaluronate lyase proves to be specific for hyaluronic acid (57). Applying this approach full recovery of the individual types in a complex synthetic mixture of 4 glycosaminoglycans is achieved (tab. 5), which indicates both the specificity and sensitivity of the procedure. However, care has to be taken with the control incubations because they scatter laser light more intensely (about 2 to 4 V) than the non-treated, native samples.

The possible interference of polynucleotides with the laser nephelometric assay was studied. Concentrations of ribosomal RNA up to 13 mg/l produce light scattering lower than 0.1 V. A similar concentration of high molecular weight DNA scatters laser light at an intensity of 3.2 V in the presence of N-cetylpyridinium chloride. Concentrations of DNA lower than 6 mg/l were without significant effect on light scattering (< 0.1 V). Thus, physiologic concentrations of DNA and RNA in body fluids, which are far below those tested here, are unlikely to affect the laser nephelometric estimation of glycosaminoglycans.

Comparison of the laser nephelometric and turbidimetric measurement of specific types of glycosaminoglycans

In relation to nephelometry the turbidimetric assay of glycosaminoglycans proves to be quite insensitive (tab. 6). Irrespective of the type of glycosaminoglycan studied, both the detection limits and the upper limits of the linear ranges are much higher for turbidimetry than for nephelometry. Typical, turbidimetrically determined calibration curves of various glycosaminoglycans are shown in figure 5. The precision of the turbidimetric measurement of glycosaminoglycans (45 mg/l chondroitin sulphate) is characterized by intra-assay (n = 20) and inter-assay (n = 10) coefficients of variation of 6% and 10%, respectively.

Tab. 6. Measurement of some glycosaminoglycans by laser nephelometry and turbidimetry at 340 nm. Comparison of detection limits (see tab. 3) and linear ranges. Analytic procedures are described in "Methods".

Type of glycosaminoglycan		Detection limit mg/l	scattered light/absorbance	Linear range mg/l
Chondroitin sulphate	nephelometry	1	0.7 V	1–100
	turbidimetry	70	0.15 A_{340}	70–250
Dermatan sulphate	nephelometry	5	0.4 V	5–125
	turbidimetry	50	0.15 A_{340}	50–500
Heparan sulphate	nephelometry	5	0.3 V	5– 90
	turbidimetry	50	0.15 A_{340}	55–270

Laser nephelometric determination of the relative concentration and composition of glycosaminoglycans in synovial fluids

Based on clinical-chemical, cytological and physicochemical criteria (56), synovial fluids (n = 38) were subdivided into those of inflammatory and non-inflammatory origin and subjected to a laser nephelometric determination of the relative concentration of glycosaminoglycans (light scattering units/ml synovial fluid). It was found that synovial fluids from non-

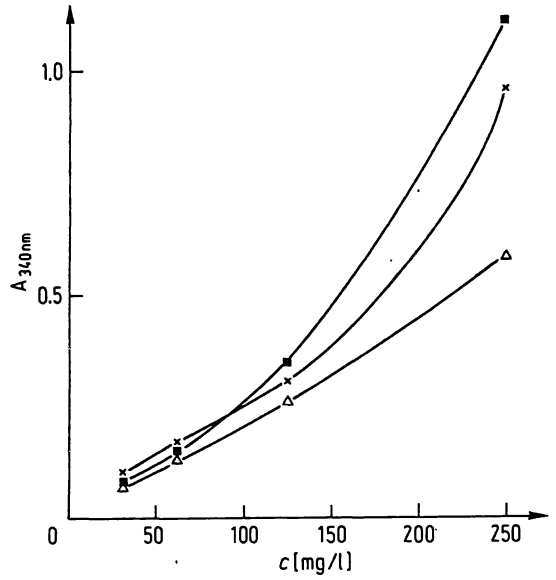

Fig. 5. Absorbance at 340 nm of the N-cetylpyridinium chloride-glycosaminoglycan complex as function of variable concentrations of chondroitin sulphate (□——□), dermatan sulphate (×——×) and highly sulphated heparan sulphate (△——△).
The conditions of incubation were as descrtibed in fig. 2, and the absorbance at 340 nm was read using a centrifugal fast analyzer.

inflammatory joint diseases contain about 4.5 times more light scattering units/ml than those of inflammatory diseases. However, a considerable interindividual variation was noted (tab. 7). A fraction of more than 0.98 of the N-cetylpyridinium chloride-induced light scattering in synovial fluid could be abolished by treatment of the specimens with hyaluronate lyase. Thus, in both pathological conditions, light scattering is due to the nearly exclusive presence of hyaluronic acid.

Tab. 7. Relative concentration of hyaluronic acid in synovial fluids from inflammatory and non-inflammatory joint diseases. The differentiation is based on cytologic, clinical-chemical and physicochemical criteria (56). 0.1 ml of synovial fluid was diluted 1 : 40 with 0.03 mol/l NaCl, and 0.5 ml of the dilution was subjected to treatment with hyaluronate lyase, filtered and incubated with N-cetylpyridinium chloride as described in "Methods".
The fraction of light scattering due to the presence of hyaluronic acid was more than 0.98 of total laser light scattering. The mean values ± S.D. are listed.

Source of synovial fluid	Number of samples	Hyaluronic acid (scattered light [V]/ml)
Inflammatory joint disease	22	175.2 ± 295.2
Non-inflammatory joint disease	16	836.4 ± 883.2

Discussion

As detailed in the present study the laser nephelometrically recorded formation of complexes between glycosaminoglycans and N-cetylpyridinium chloride can be utilized under certain conditions for a reliable measurement of total and specific glycosaminoglycans. The method proves sensitive, precise, inexpensive and relatively simple, and is therefore probably widely applicable. In comparison to the previously described turbidimetric measurement of glycosaminoglycans using either N-cetylpyridinium chloride (48, 49) or cetyltrimethylammonium bromide (47) as complexing agents, the sensitivity of the nephelometric method is superior; this is also the case in the immunologic measurement of proteins with the two procedures. Therefore, the laser nephelometric principle might also be advantageous for the determination of glycosaminoglycans in low concentrations in tissue extracts (e.g. biopsy specimens), cell culture extracts, or in fluids available only in small amounts (e.g. amniotic fluid). Laser nephelometry has recently proved to be useful in the estimation of glycosaminoglycans in human serum (51). Compared with the quantitation of glycosaminoglycans by determination of uronic acid, laser nephelometric measurement is much more simple, has an even higher sensitivity and is independent of the type of uronic acid (which is known to influence the carbazole reaction (60)).

The laser nephelometric method is based on the measurement of glycosaminoglycans by virtue of their polyanionic structure. Some of the drawbacks of the proposed method are related to the complicated, physico-chemically carefully explored interactions of glycosaminoglycans with quaternary ammonium salts (42, 43, 63). The complex formation is known to be dependent on the ionic strength and the pH of the solution (42). A precipitating complex does not occur with keratan sulphate in the presence of excess N-cetylpyridinium chloride (42, 43, 64). Thus, the latter type of glycosaminoglycans is not measurable with the nephelometric method (fig. 2). Furthermore, small molecular weight fractions of glycosaminoglycans (< octasaccharides) do not form complexes with the organic cations and, hence, will be not detectable. This fact seems to be noteworthy since glycosaminoglycans in urine and serum, probably in other body fluids as well, are partly low molecular weight breakdown products of the high molecular weight aggregates present in connective tissue (41). The most serious complication of the method arises from the dependency of the complex formation not only on the chain length but also on the degree of sulphation or charge density of the glycos-

aminoglycan; an example of this is presented in figure 4. The effect of the anionic structure of the glycosaminoglycan on the intensity of light scattering in the presence of quaternary ammonium salts raises insuperable difficulties in the selection of appropriate reference glycosaminoglycans (calibration curves) necessary for the calculation of the concentrations of glycosaminoglycans in biological materials. Therefore it is more reliable to determine the relative fractions of various glycosaminoglycans (tab. 7) rather than absolute concentrations. Due to the arbitrary choice of glycosaminoglycan standards, the latter can be only rough estimates. On the other hand, changes of the relative amount and composition of glycosaminoglycans are detected with sensitivity by the nephelometric method. The specificity of the N-cetylpyridinium chloride-induced light scattering in synovial fluid for glycosaminoglycans is proved by its complete abolition when glycosaminoglycans are destroyed with hyaluronate lyase. In serum, there is a high degrec of laser light scattering due to unspecific reactions of N-cetylpyridinium chloride with serum components other than glycosaminoglycans (51). In contrast, synovial fluids did not contain appreciable concentrations of non-glycosaminoglycan molecules that were able to form light scattering complexes with the organic cations. Even high concentrations of DNA and RNA do not interfere with the laser nephelometry of glycosaminoglycans.

The specificity and efficiency of the chemical and enzymatic, laser nephelometrically monitored differentiation of complex glycosaminoglycan mixtures is substantiated by the complete recovery of the individual compounds (tab. 5). The time required for the development of optimal light scattering, between 14 and 18 h, delays the performance of the assay in its present form. However, light scattering can be read reproducibly at 3 h if a reduction of sensitivity is accepted. Based on the concentration-dependent differences in the time course of light scattering shown in figure 1 we currently evaluate a peak rate modification of the kinetic nephelometric assay instead of the fixed-time method described here, in order to prevent prolonged incubation times.

Using the laser nephelometric procedure we demonstrate in synovial fluids of non-inflammatory joint diseases a much higher light scattering due to hyaluronic acid than in those of inflammatory origin. This result accords with previous reports, which describe significantly elevated concentrations of hyaluronate in degenerative joint diseases in comparison to inflammatory articular lesions (e.g. rheumatoid arthritis) (55, 56). Therefore, the laser nephelometric assay of synovial hyaluronic acid is suggested as an additional, simple and sensitive laboratory aid in the discrimination between both pathogenetic types of articular effusions.

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